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## Original article

# Isolation and identification of virulent bacteria from forensic flies as mechanical vectors associated with a rabbit carcass

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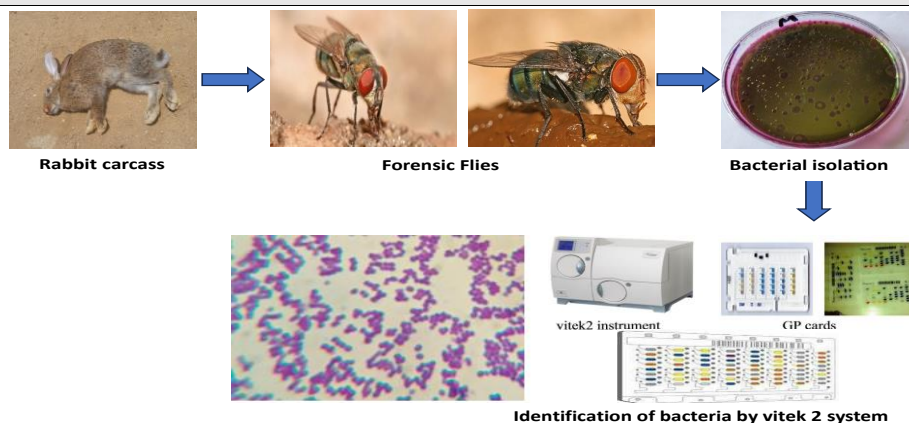
*Escherichia coli*

*Klebsiella pneumoniae ssp pneumoniae*

### ABSTRACT

Flies can transfer a remarkable range and variety of pathogens including bacteria, fungi, viruses, and parasites. Herein, forensic flies' succession associated with the decomposition stages of a rabbit carcass, bacterial variety, and the total bacterial load associated with the external body surface of these forensic flies were investigated. A total of 340 adult insects were collected from rabbit carcass during decomposition stages, most of them are flies. Fresh, bloated, and decay stages attracted the greatest numbers and highest diversity of flies species. Moreover, *Chrysomya albiceps* and *Musca domestica* were recorded in all decaying stages. Several pathogenic bacteria were isolated from the flies' body surface on selective and differential media and identified to the species level by using the Vitek2 system. The high-ranking bacterial count and diversity were isolated from *Chrysomya albiceps*, *Wohlfartia magnifica*, and *Musca domestica*, respectively during the decomposition stages. The results revealed that Enterobacteriaceae was the most dominant isolated bacteria from the collected flies. The Percentage of bacterial incidence presented that *E. coli* was the most detected pathogen (40%) followed by *Staphylococci*. (35%), *Pseudomonas aeruginosa* and *Klebsiella pneumoniae ssp pneumoniae* (10% each), *Proteus mirabilis* (5%). The present study provides a steppingstone for studying the microbiota diversity of flies causing myiasis. The prevalence of *E. coli* gained in this survey is a cause for public health concern as this bacterium has been incriminated in cases of gastroenteritis. Accordingly, efforts are required to raise awareness among all people about personal hygiene and clean surfaces subjected to flies.

### Graphical abstract



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## 1. Introduction

Forensic entomology assesses the ecological as well as biological characteristics of arthropod fauna that colonize novel rhizosphere habitats. It is related to forensic research [1]. In the absence of vertebrate scavengers, animal carcasses offer an incredibly rich variety of insects that engage with anaerobes to hasten the decomposition process [2]. Flies represent the initial insects to occupy a dead body. The species of flies implicated vary depending on location, although multiple studies have shown that the primary species involved belong to a restricted number of families: Calliphoridae, Sarcophagidae, and Muscidae [3, 4]. As bacteria are responsible for many characteristics of decomposition (including fresh, bloat, activity, advanced skeletonization, and skeletonization), they may regulate insect response to collect species that are beneficial to their survival while resisting species that are harmful [5, 6]. Temperature, wind, moisture, geographical region, the make-up of the fauna, the succession order, and the period of insect existence are only a few of the many factors that affect the process of decomposition. When native climatological data are available, the influences of the indigenous fauna can be used to estimate the postmortem interval (PMI) [7, 8]. Knowing the roles of microbes in the degradation of carrion and how they pertain to post-mortem interval measurement requires knowing that bacteria are the main decomposers. According to Janaway et al. [9] autolysis and bacterial activity within the tissues both contribute to the first disintegration of tissues. Internal bacteria, such as those from the gastrointestinal GI tract, spread as the tissues break down. Because of the lack of oxygenated blood, the tissue's loss of redox potential is probably what causes the transition from aerobic to anaerobic organisms. Flies have been recognized as mechanical carriers of more than 130 bacterial, viral, and parasitic infections [10, 11, 12]. In hospitals and restaurants, flies are documented as active carriers of virulent bacteria [13]. The most common pathogenic bacteria transmitted by flies include *Klebsiella spp.* [14], *Salmonella* [15], *Pseudomonas aeruginosa* [16], *Campylobacter jejuni* [17], *Edwardisella spp.* [18]. Houseflies can acquire and harbor the contagious SARS-CoV-2 virus for up to 24 hr. after exposure, they are capable of mechanically transmitting SARS-CoV-2 genomic RNA to the environment [11]. Consequently, this study aims to (1): Determine flies succession associated with the decomposition stages of a rabbit carcass, (2): Determine bacterial variety, and the total bacterial load associated with the external body surface of forensic flies, and (3): Identify most detected bacteria using Vitek system.

## 2. Materials and methods

### Chemicals and reagent

Analytical purity chemicals used in this work were sourced from Sigma-Aldrich Chemical Co (St. Louis Missouri, 63103, USA). Eosin Methylene Agar (EMB), MacConkey Agar, and Baird Parker were from (Oxoid Ltd. and Termo Scientific, UK) and Plate Count Agar (PC) was from (Scharlau Chemie S.A, Barcelona, Spain).

### Study animal and experimental site

The first part of the study was conducted between July and August 2022 at the botanical garden of the animal house, Zoology and Entomology Department, Faculty of Science, AL- Azhar University. One rabbit (*Lepus cucinulus*) 2 kg. was purchased from local markets in Cairo. The experiments on selected rabbit were conducted according to the Institutional Animal Ethics Committee of Al-Azhar University. The rabbit was killed with a blow on the head, and the carcass was quickly placed into mesh coops to avoid scavenging by large animals and permitted to decay.

### Collection of flies

A hand net was employed to trap flies from the rabbit carcass. The carcass was revisited daily to establish the extent of the decomposition processes. The digital camera was used to record images of the decaying rabbit carcass. Identification and taxon classification of flies were carried out according to Greenberg [19], Mosallam [20], Shaumar et al. [21], Whitworth [22], Carvalho and Mello-Patiu [23]. Both humidity and temperature were measured daily.

### Bacteria isolation from collected flies' outer surfaces

This part of the study was conducted at the Botany and Microbiology Department, Faculty of Science, Al-Azhar University (Girls Branch). The different flies were aseptically placed in 5ml of sterilized saline solution and shaken well for 10 minutes. All samples were serially diluted to  $10^{-3}$ . Because the associate assumed the inclusion of coliforms and other fastidious organisms, a general purpose enriched medium (plate count agar medium), selective and differential medium (Eosin methylene blue agar and MacConkey agar), as well as Baird Parker agar medium were used throughout this work. All media used were weighed and prepared according to the Manufacturer's instructions and were autoclaved at 121°C for 15 minutes. All plates were labeled on the top, and 100 microliters of  $10^{-3}$  dilution were pipetted into plates containing different media. The plates were shaken gently as immediately as the agar was poured to retain the bacteria separated throughout growth. The medium was allowed to solidify before being incubated at 37°C for 24 hr. and up to 48 hr.

### Purification and identification of bacterial isolates

The purification of the most common bacterial isolates was carried out by the agar streak method on the agar surface of plates containing the same isolation medium and incubated at 35 °C for 24 hr. The isolated bacteria were preliminarily identified based on colony morphology on the selective and differential media [15]. Purity was checked up by microscopic investigation using Gram stain. The Pure isolates were sub-cultured on slants of PC agar medium and kept for further investigation. The most common bacterial isolates were completely identified to the species level by using the Vitek- 2 system at Al-Mokhtabar laboratory, Cairo, Egypt.

## 3. Results

### Recording the decomposition stages and environmental conditions

Regarding the decomposition stages of rabbit carcass, the fresh stage of decomposition began with the death and lasted 12 hr. postmortem. The beginning of the bloated stage was on day 1 postmortem and lasted until day 2. The ending of the bloated stage and the beginning of the active decay stage was confirmation of the liquefaction process. Evidence of liquefaction first occurred on the fourth day. The advanced decay began on day 4 when the flesh of the rabbit carcass was removed from the head, limbs, and anus. The final stage of decomposition is the dry stage which was reached on day 7 and lasted until day 10 and was characterized by little odor and hardened, dried, exposed bone (**Table 1& plate 1**).

#### Succession of forensic flies on a rabbit carcass

In the current work, a total of 340 adult insects representing 3 orders, 7 families and 9 species were collected during decomposition stages of rabbit carcass. As illustrated in **table (2)**, only 3 Diptera species appeared around the fresh carcass, 2 *Chrysomya albiceps* flies (family Calliphoridae), 2 *Musca domestica* flies (family Muscidae), 3 *Wohlfartia magnifica* flies (family Sarcophagidae), were collected at the second decaying stage and at bloated one another insect orders began to be attracted to the carcass site beside the flies. Forty-six *Chrysomya albiceps* and 2 *Chrysomya megacephala* (family Calliphoridae) as well as 25 *Musca domestica*, 2 *Sarcophaga carnaria* and 23 *Wohlfartia magnifica*(family Sarcophagidae), Six *Piophilidae casei* (family Piophilidae). At the decay stage, two species of Calliphoridae were collected, *Chrysomya albiceps* 39, and *Calliphora sp 1*.

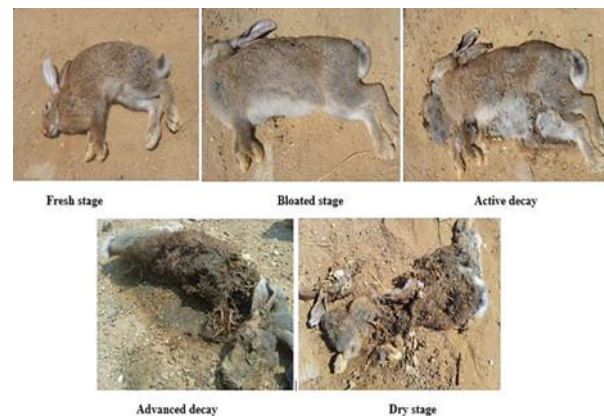
From family Sarcophagidae only one species *Wohlfartia magnifica* accounted for 7 flies, while family Muscidae was represented by 41 *Musca domestica*. Finally, 8 *Piophilidae casei* flies from the family Piophilidae were collected. The dry stage of rabbit was categorized by the presence of 11 *Chrysomya albiceps* flies, 11 *Musca domestica*, and coleopteran insects (Families Histeridae and Dermstidae) were represented by 4 and 17 individuals collected from the rabbit carcass, while there were 90 ants belonging to order Hymenoptera, family Formicidae at the different decaying stages followed the fresh one.

#### Isolation of bacteria

In the current investigation, four types of media (Plate count agar, Eosin methylene blue (EMB), MacConkey agar (MAC) and Baird parker (B.P) were used for bacterial isolation intent. The obtained results showed that all the collected flies from the most infested decomposition stages (fresh, bloated, and decay) of rabbit carcass were contaminated with varying levels of bacterial counts. From the external surface of *Chrysomya albiceps* flies which were found in all decomposition stages (fresh, bloated, and decay stages), both Gram -positive and Gram-negative bacteria were isolated on the four used media, table (3) and plate 2 (A-C).

**Table (1): Decomposition stages of rabbit carcass and environmental conditions.**

Decomposition stages	Days of postmortem	Temperature (°C)		Average relative humidity %
		Maximum	Minimum	
Fresh	0 -12 hr.	37	24	47
Bloated	1-2	36	25	46
Active decay	2-3	35	25	47
Advanced decay	4-6	39	25	49
Dry	7-10	39	25	48



**Plate (1): Decomposition stages of rabbit carcass**

Additionally, it was obvious from **Table (4)** and plate (5), that the *Chrysomya megacephala* was present in the bloated stage only. The isolated bacteria belonged to the Enterobacteriaceae and *Staphylococcus*. On the other hand, *Calliphora sp* was detected at the decay stage only and all the isolated bacteria from this fly were Gram-negative Enterobacteriales (table 5, plate 6). Interestingly, *Musca domestica* flies were found around all stages of rabbit decaying. The results obtained from these screenings showed that *Musca domestica* over 340 investigated flies was the most contaminated fly by bacterial isolates (**table 6, plate 5 A-C**). Interestingly, the fly species, *Sarcophaga carnaria* was found in the bloated stage only. The data illustrated in **table (8)**, indicated that most encountered bacterial isolates from the external surface of *Sarcophaga carnaria* fly were gram-negative bacteria (plate 7).

Regarding the incidence of *Wohlfartia magnifica*, it was presented in the first three stages of decaying. The results listed in **table (7)** clarified that *Wohlfartia magnifica* was the extremely contaminated fly by bacterial isolates over the 340 investigated flies (plate 6 A-C).

The data listed in (**table 9**) showed the absence of *Piophilidae casei* in the fresh stage and was present in both the bloated and decay stages. At bloated stage, only Gram-positive *Staphylococci* were encountered, while at the decay stage both Gram-positive and Gram -negative isolates were obtained.

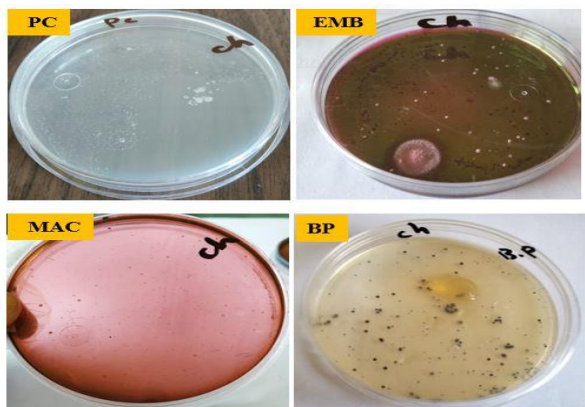
**Identification of bacteria by Vitek 2 system**

Analytical Profile Indexes from the Vitek 2 system were used to detect bacterial species. Vitek 2 system successfully identified 40 bacterial isolates belonging to 5 different genera. The identified bacteria included *Staphylococcus epidermidis*, *Staphylococcus hominis*, *Staphylococcus warneri*, *Staphylococcus sciuri*, *Escherichia coli*, *Klebsiella pneumoniae ssp. Pneumonia*, *Pseudomonas aeruginosa* and *proteus mirabilis*. Moreover, *E. coli* was the most prevalent bacterial isolate and represented 40%.

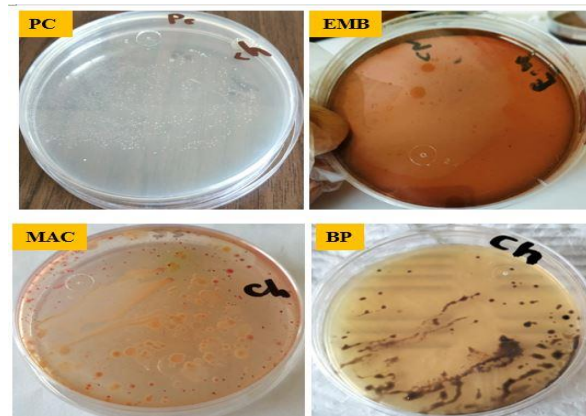
Additionally, Figure 1 depicts the percentage incidence of virulent bacterial species isolated from forensic flies associated with different decomposition stages of rabbit carcass. The ID profile assurance was very good. Vitek 2 Systems identify an organism by using a methodology based on the characteristics of the data and knowledge about the organism and reactions being analyzed. In this identification system, sufficient data have been collected from known strains to estimate the typical reactions of the claimed species to a set of discriminating biochemicals (Table 10- 17).

**Table (2): Insects associated with decomposition stages of the rabbit carcass.**

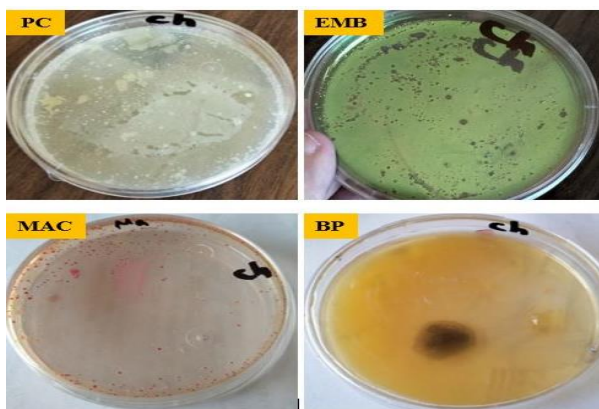
Order	Family	Species	Decomposition stages/Days postmortem				Total
			Fresh	Bloated	Decay	Dry	
			0-1	1-2	2-6	7-10	
Diptera	Calliphoridae	<i>Chrysomya albiceps</i>	2	46	39	11	98
		<i>Chrysomya megacephala</i>	0	2	0	0	2
		<i>Calliphora sp</i>	0	0	1	0	1
	Muscidae	<i>Musca domestica</i>	2	25	41	11	79
	Sarcophagidae	<i>Wohlfartia magnifica</i>	3	23	7	0	33
		<i>Sarcophaga carinaria</i>	0	2	0	0	2
	Piophilidae	<i>Piophila casei</i>	0	6	8	0	14
Coleoptera	Dermstidae	<i>Dermestes maculatus</i>	0	0	4	0	4
	Histeridae	<i>Hister sp</i>	0	1	16	0	17
Hymenoptera	Formicidae		0	60	1	29	90
Total						340	



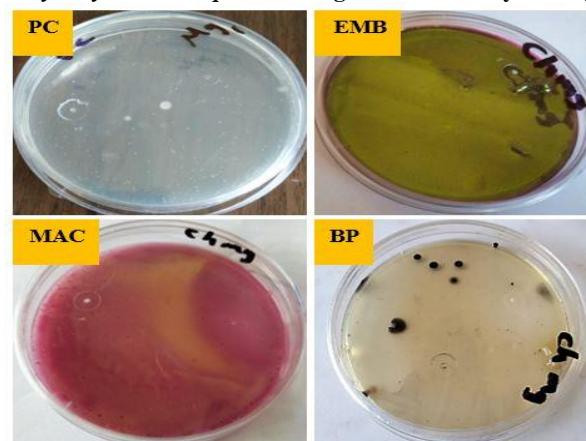
**Plate (2A): Different bacterial colonies isolated from *Chrysomya albiceps* during the fresh stage.**



**Plate (2 C): Different bacterial colonies isolated from *Chrysomya albiceps* during the decay stage**



**Plate (2B): Different bacterial colonies isolated from *Chrysomya albiceps* during the bloated stage.**



**Plate (3): Different bacterial colonies isolated from *Chrysomya megacephala* during the bloated stage.**

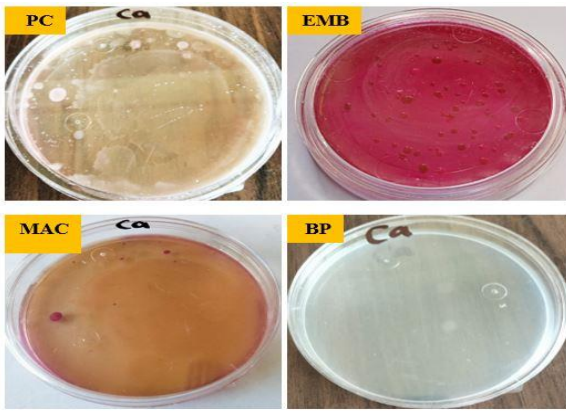


Plate (4): Different bacterial colonies isolated from *Calliphora sp* during the decay stage.

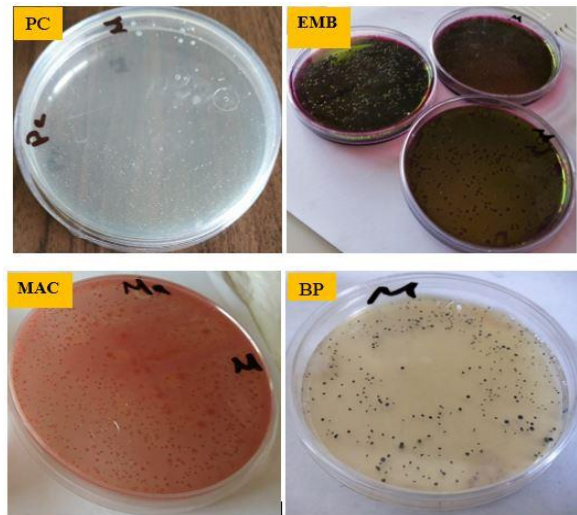


Plate (5B) Different bacterial colonies isolated from *Musca domestica* during the bloated stage.

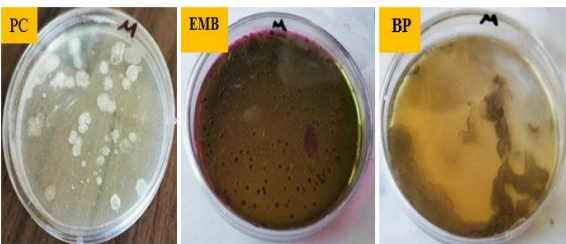


Plate (5 A) Different bacterial colonies isolated from *Musca domestica* during the fresh stage.

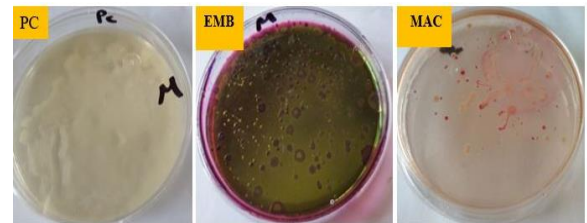


Plate (5C) Different bacterial colonies isolated from *Musca domestica* during the decay stage.

Table (3): Total bacterial count isolated from *Chrysomya albiceps* during the decomposition stages of rabbit carcass.

Stages of decomposition	No. of <i>Ch. albiceps</i>	Bacterial total count Cfu/ml on each media			
		P.C	E.M. B	MAC	B.P
Fresh	2	$300 \times 10^{-3}$	$250 \times 10^{-3}$	$290 \times 10^{-3}$	$1 \times 10^{-3}$
Bloated	46	$85 \times 10^{-3}$	$112 \times 10^{-3}$	$66 \times 10^{-3}$	$98 \times 10^{-3}$
Decay	39	$200 \times 10^{-3}$	$64 \times 10^{-3}$	$42 \times 10^{-3}$	$59 \times 10^{-3}$

Key: PC – Plate count Agar, E.M.B- Eosin Methylene Blue Agar, MAC - MacConkey Agar, BP- Baird Parker Agar

Table (4): Total bacterial count isolated from *Chrysomya megacephala* during the decomposition stages of rabbit carcass.

Stages of decomposition	No. of <i>Ch. megacephala</i>	Bacterial total count Cfu/ml on each media			
		P.C	E.M. B	MAC	B.P
Fresh	-	-	-	-	-
Bloated	2	TNTC	TNTC	TNTC	$12 \times 10^{-3}$
Decay	-	0	-	-	-

Key: PC – Plate count Agar, E.M.B- Eosin Methylene Blue Agar, MAC - MacConkey Agar, BP- Baird Parker Agar

Table (5): Total bacterial count isolated from *Calliphora sp* during the decomposition stages of rabbit carcass.

Stages of decomposition	No. of <i>Calliphora sp.</i>	Bacterial total count Cfu/ml on each media			
		P.C	E.M. B	MAC	B.P
Fresh	-	-	-	-	-
Bloated	-	-	-	-	-
Decay	1	$140 \times 10^{-3}$	$90 \times 10^{-3}$	$11 \times 10^{-3}$	-

Key: PC – Plate count Agar, E.M.B- Eosin Methylene Blue Agar, MAC - MacConkey Agar, BP- Baird Parker Agar

Table (6): Total bacterial count isolated from *Musca domestica* during the decomposition stages of rabbit carcass.

Stages of decomposition	No. of <i>Musca domestica</i>	Bacterial total count Cfu/ml on each media			
		P.C	E.M. B	MAC	B.P
Fresh	2	$128 \times 10^{-3}$	$110 \times 10^{-3}$	-	$15 \times 10^{-3}$
Bloated	25	$100 \times 10^{-3}$	$300 \times 10^{-3}$	$295 \times 10^{-3}$	$7 \times 10^{-3}$
Decay	41	TNTC	$180 \times 10^{-3}$	-	-

Key: PC – Plate count Agar, E.M.B- Eosin Methylene Blue Agar, MAC - MacConkey Agar, BP- Baird Parker Agar

**Table (7): Total bacterial count isolated from *Wohlfartia magnifica* during the decomposition stages of rabbit carcass**

Stages of decomposition	No. of <i>Wohlfartia magnifica</i>	Bacterial total count Cfu/ml on each media			
		P.C	E.M. B	MAC	B.P
Fresh	3	TNTC	$60 \times 10^{-3}$	-	$8 \times 10^{-3}$
Bloated	23	$167 \times 10^{-3}$	$90 \times 10^{-3}$	$50 \times 10^{-3}$	$5 \times 10^{-3}$
Decay	7	$150 \times 10^{-3}$	$102 \times 10^{-3}$	$65 \times 10^{-3}$	$7 \times 10^{-3}$

Key: PC – Plate count Agar, E.M.B- Eosin Methylene Blue Agar, MAC - MacConkey Agar, BP- Baird Parker Agar

**Table (8): Total bacterial count isolated from *Sarcophaga carnaria* during the decomposition stages of rabbit carcass.**

Stages of decomposition	No. of <i>Sarcophaga carnaria</i>	Bacterial total count Cfu/ml on each media			
		P.C	E.M. B	MAC	B.P
Fresh	-	-	-	-	-
Bloated	2	$250 \times 10^{-3}$	$108 \times 10^{-3}$	$17 \times 10^{-3}$	$2 \times 10^{-3}$
Decay	-	-	-	-	-

Key: PC – Plate count Agar, E.M.B- Eosin Methylene Blue Agar, MAC - MacConkey Agar, BP- Baird Parker Agar



Plate (6 A) Different bacterial colonies isolated from *Wohlfartia magnifica* during the fresh stage.

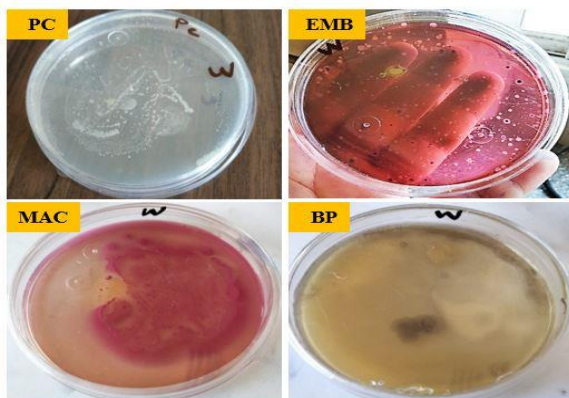


Plate (6 B) Different bacterial colonies isolated from *Wohlfartia magnifica* during the bloated stage.

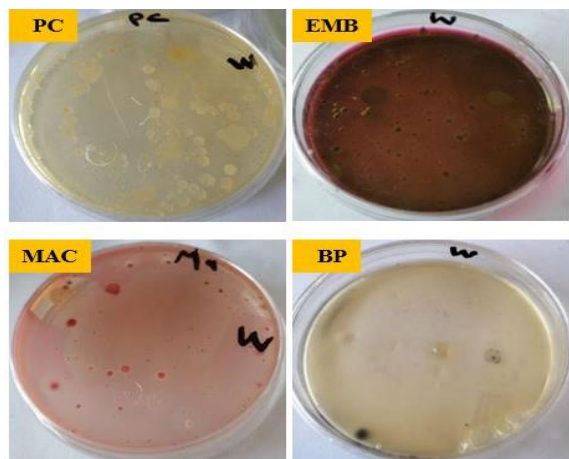


Plate (6 C) Different bacterial colonies isolated from *Wohlfartia magnifica* during the decay stage.

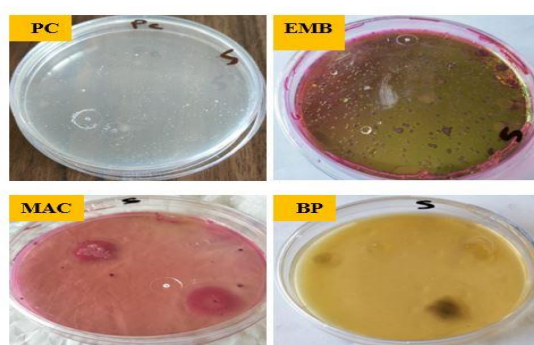


Plate (7) Different bacterial colonies isolated from *Sarcophaga carnaria* during a bloated stage.



Plate (8): Different bacterial colonies isolated from the external body surface of *Piophila casei* during the decay stage.

**Percentage of Bacterial Incidence**

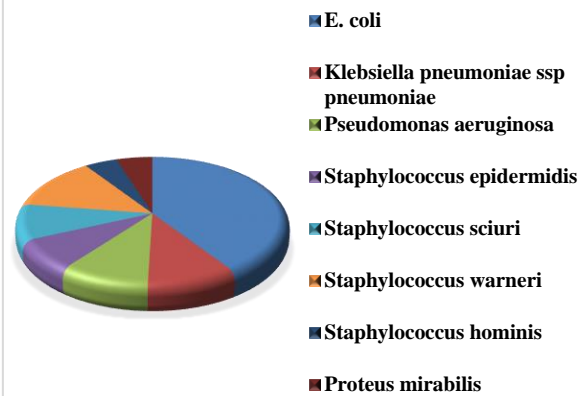


Figure (1): Percentage incidence of virulent bacterial species isolated from forensic flies associated with different decomposition stages of rabbit carcass.



**Table (13): Identification data of *Pseudomonas aeruginosa***

Code		Probability			Card		Assurance			Analysis time			Identified bacteria				
M5 ( <i>Musca</i> )		98%			GN		Very good Identification			4.08 hr.			<i>Pseudomonas aeruginosa</i>				
2	APPA	-	3	ADO	+	4	PyrA	+	5	IARL	-	7	dCEL	-	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	-
40	ILATk	-	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	+
46	GlyA	+	47	ODC	-	48	LDC	+	53	IHISa	-	56	CMT	+	57	BGUR	+
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILAT	-			

**Table (14): Identification data of *Klebsiella pneumoniae ssp pneumoniae***

Code		Probability			Card		Assurance			Analysis time			Identified bacteria				
Pi (mg4) <i>Piophila</i>		99%			GN		Very good Identification			3.87 hr.			<i>Klebsiella pneumoniae ssp pneumoniae</i>				
1	APPA	-	2	ADO	+	3	PyrA	-	4	IARL	-	5	dCEL	+	6	BGAL	+
7	H2S	-	8	BNAG	-	9	AGLTp	-	10	dGLU	+	11	GGT	+	12	OFF	+
13	BGLU	+	14	dMAL	+	15	dMAN	+	16	dMNE	+	17	BXYL	+	18	BAlap	-
19	ProA	-	20	LIP	-	21	PLE	+	22	TyrA	+	23	URE	+	24	dSOR	+
25	SAC	+	26	dTAG	+	27	dTRE	+	28	CIT	+	29	MNT	+	30	5KG	-
31	ILATk	+	32	AGLU	-	33	SUCT	+	34	NAGA	-	35	AGAL	+	36	PHOS	+
37	GlyA	-	38	ODC	-	39	LDC	+	40	IHISa	-	41	CMT	-	42	BGUR	-
43	O129R	+	44	GGAA	-	45	IMLTa	-	46	ELLM	-	47	ILATa	-	48		

**Table (15): Identification data of *Escherichia coli***

Code		Probability			Card		Assurance			Analysis time			Identified bacteria				
M3 ( <i>Musca</i> )		99%			GN		Very good Identification			3.87 hr.			<i>Escherichia coli</i>				
1	APPA	-	2	ADO	-	3	PyrA	-	4	IARL	-	5	dCEL		6	BGAL	+
7	H2S	-	8	BNAG	-	9	AGLTp	-	10	dGLU	+	11	GGT	-	12	OFF	+
13	BGLU	-	14	dMAL	+	15	dMAN	+	16	dMNE	+	17	BXYL	-	18	BAlap	-
19	ProA	+	20	LIP	-	21	PLE	-	22	TyrA	+	23	URE	-	24	dSOR	+
25	SAC	-	26	dTAG	-	27	dTRE	+	28	CIT	-	29	MNT	-	30	5KG	-
31	ILATk	+	32	AGLU	-	33	SUCT	+	34	NAGA	-	35	AGAL	+	36	PHOS	-
37	GlyA	-	38	ODC	+	39	LDC	+	40	IHISa	-	41	CMT	+	42	BGUR	+
43	O129R	+	44	GGAA	-	45	IMLTa	+	46	ELLM	-	47	ILATa	-	48		

**Table (16): Identification data of *Escherichia coli***

Code		Probability			Card		Assurance			Analysis time			Organism				
S2 ( <i>Sarcophaga</i> )		99%			GN		Very good Identification			3.83 hr.			<i>Escherichia coli</i>				
1	APPA	-	2	ADO	-	3	PyrA	-	4	IARL	-	5	dCEL		6	BGAL	+
7	H2S	-	8	BNAG	-	9	AGLTp	-	10	dGLU	+	11	GGT	-	12	OFF	+
13	BGLU	-	14	dMAL	+	15	dMAN	+	16	dMNE	+	17	BXYL	-	18	BAlap	-
19	ProA	+	20	LIP	-	21	PLE	-	22	TyrA	+	23	URE	-	24	dSOR	+
25	SAC	-	26	dTAG	-	27	dTRE	+	28	CIT	-	29	MNT	-	30	5KG	-
31	LLATk	+	32	AGLU	-	33	SUCT	+	34	NAGA	-	35	AGAL	+	36	PHOS	-
37	GlyA	-	38	ODC	+	39	LDC	+	40	IHISa	-	41	CMT	+	42	BGUR	+
43	O129R	+	44	GGAA	-	45	IMLTa	+	46	ELLM	-	47	ILATa	-	48		

**Table (17): Identification data of *Proteus mirabilis***

Code		Probability			Card		Assurance			Analysis time			Organism				
M1 ( <i>Musca</i> )		98%			GN		Very good Identification			5.00 hr.			<i>Proteus mirabilis</i>				
1	APPA	-	2	ADO	+	3	PyrA	-	4	IARL	-	5	dCEL	-	6	BGAL	+
7	H2S	-	8	BNAG	-	9	AGLTp	-	10	dGLU	+	11	GGT	-	12	OFF	+
13	BGLU	-	14	dMAL	+	15	dMAN	+	16	dMNE	+	17	BXYL	-	18	BAlap	-
19	ProA	+	20	LIP	-	21	PLE	-	22	TyrA	+	23	URE	-	24	dSOR	+
25	SAC	+	26	dTAG	-	27	Dtre	+	28	CIT	-	29	MNT	-	30	5KG	-
31	ILATk	-	32	AGLU	-	33	SUCT	+	34	NAGA	-	35	AGAL	+	36	PHOS	-
37	GlyA	+	38	ODC	-	39	LDC	+	40	IHISa	-	41	CMT	+	42	BGUR	+
43	O129R	+	44	GGAA	-	45	IMLTa	-	46	ELLM	-	47	ILATa	-	48		



#### 4. Discussion

The present investigation provides a steppingstone for studying the bacteria diversity of flies causing myiasis's associated with rabbit carcass. Forensic insects have indeed been explored on several animal carcasses, including cats [24], dog [25], pigs [26], guinea pigs [27], mice [22], rabbit and dog carcasses [28]. More interestingly, **Azwandi et al.** [29] evaluated the arthropod phyla diversity on rat, rabbit, and long-tail monkey carcasses and found differences in the number of species collected. Similar variance was also discovered in rabbit carcasses in the current investigation. The outcomes of this study revealed that while the Calliphoridae were more abundant during the earlier stages of decomposition, the Sarcophagidae were predominant during the later stages. These results are consistent with those obtained by **De-Carvalho and Linhares**, [30] using pig carcass and **Zeariya et al.**, [28] using rabbit and dog carcasses. The results indicated *Musca domestica*, *Chrysomya albiceps* and, *Wohlfartia magnifica* were the most common flies during the decomposition stages of rabbit carcass. Our results were consistent with previous studies by **Payne**, [31]; **Zeariya et al.**, [28] that reported Blow flies, especially *Chrysomya albiceps* played a fundamental role in the carcass decomposition. These flies confirm their role as major factors in carcass decomposition. A study by **Turner**, [32] indicated that every stage of decomposition appeals to different species due to the physical and chemical parameters surrounding the carrion. Moreover, according to **Kaiko and Stappenbeck** [33], the successful breakdown of animal tissues requires complex microbial interactions and successions, which can be impacted by environmental factors. Insects and microbes repeatedly engage in persistent activity during this process, which is followed by an active decay [34]. Also, enteric soil bacteria play a significant role in the decomposition of animal tissues [35]. According to **Mohammed et al** [36], the advanced deterioration stage begins as soon as the majority of the muscoid fly larvae have left the carcass. The internal organ structure as a whole has been whittled down to a goeey mush. Following consumption and drying of this material, the dry residues stage begins. Many factors, including insect activity and abundance, location, temperature, humidity, rainfall, habitat, season, and the size and kind of the carcass, affect how long each of these stages lasts. Studies demonstrating the significant impact that flies have on the decomposition process were effective in measuring both the availability of carrion to insects and the absence of insects from carrion. The carcass eventually broke down through a well definite phase of decay, attracting flies, beetles, ants, and a variety of other insects [37]. Moreover, the succession patterns of carrion-arthropods, which show how the nature and composition of fauna vary throughout the duration of decay, is another technique [38].

Because authors assumed the inclusion of coliforms and other fastidious organisms, a general purpose enriched medium (plate count agar medium), selective and differential medium for Gram-Negative bacteria (Eosin methylene blue agar and MacConkey agar), as well as (Baird Parker agar medium) for Gram-positive bacteria, were used throughout this work. One of the most

common approaches for assessing microbial diversity is selective and differential plating, followed by viable counts. These approaches, which are quick and affordable, provide information about the culturable segment of the microbial population. Culturing on selective media may also be effective for identifying and isolating essential microorganisms with certain properties (beneficial or pathogenic) in a less expensive manner [39]. Early investigations of microbial communities relied heavily on traditional cultural practices and were typically focused on infective strains [40, 41]. Numerous in-depth assessments employing culture-independent methods have been established in recent years to examine bacterial populations in many insects [42, 43, 44]. Flies are especially interesting because many of them reproduce in decomposing organic debris, which is colonized by a variety of microbes. The choice of appropriate growth media and the stipulation of growth circumstances are variables that restrict the adoption of these approaches (temperature, pH, and aeration). Moreover, the outcomes of this study indicated that, all collected flies were highly contaminated with different pathogenic bacteria. The bacteria that were identified throughout this work were *Staphylococcus epidermidis*, *Staphylococcus hominis*, *Staphylococcus warneri*, *Staphylococcus sciuri*, *Escherichia coli*, *Klebsiella pneumonia ssp. pneumonia* *Pseudomonas aeruginosa* and *Proteus mirabilis*. Our results have implications for increased risk of disease due to fly transmission of pathogenic *E. coli*. The obtained results are in perfect harmony with the results obtained by **Mohammed et al.**, [45] who conducted an ecological study on zoonotic bacteria distributed by flies in cow farms and discovered that the most common bacterial isolates concluded were *Escherichia coli*, *Staphylococcus aureus*, and *Enterobacter*, *Staphylococci*, *Klebsiella sp.*, *Salmonella spp.*, *Shigella spp.*, and *Proteus spp.* Also, **Fouda et al.**, [44] isolated and identified 11 bacterial strains from the adults of *Musca domestica*, *Chrysomya albiceps* and *Lucilia sericata* associated with different carions. The incidence of *E. coli* and other *Enterobacteriales* in this study is a cause for public health concern as this bacterium has been implicated in cases of gastroenteritis. *Staphylococcus spp.*, *Bacillus spp.*, and *E. coli* are recognized for producing powerful enterotoxins that cause a rapid onset of disease within three to four hrs., with nausea, vomiting, and diarrhea being the most common symptoms [46].

Our results support previous studies that identified *Enterobacteriales* as the most dominant species isolated from several insects [47, 48]. Despite their predominance, the *Enterobacteriales* are not the sole taxon found on the external surface of collected flies. Also, **Förster et al.**, [49] conducted a laboratory experiment on synanthropic flies (such as *Musca*, *Sarcophaga*, *Calliphora*, *Fannia*, *Lucilia*, and *Stomoxys*) as carriers of virulent microbes, and concluded that the various fly species were connected to foodborne and other infections. The house fly, *Musca domestica*, has long been thought of as a possible vector for epidemics since its inception, [50]. Additionally, **Butler et al.**, [51] investigated how harmful germs are transported by wild Florida house flies (*Musca domestica*). *Acinetobacter baumannii*, *Bacillus pumilus*,

*Cronobacter sakazakii*, *Methylobacterium persicinum*, and *Staphylococcus sciuri* were five of the newly discovered bacteria for house flies. Others such as *Bacillus cereus*, *B. thuringiensis*, *Escherichia coli* O157:H7, *Shigella dysenteriae*, *Staphylococcus saprophyticus*, and *Staphylococcus xylosus*, have also been linked to house flies in the past. Also, **Choo et al.**, [52] studied the isolation of *Campylobacter* and *Salmonella* from *Musca domestica* and stated that, the insects particularly house flies, and cockroaches, have been linked to the spread of pathogens in farms and in human epidemic diseases, including *Salmonella* spp and *Campylobacters*.

The *Chrysomya megacephala* was studied by **Chaiwong et al.**, [53], and the two bacteria that were most isolated from *C. megacephala* were *streptococci* and *staphylococci*. *C. megacephala* has the potential to transmit harmful intestinal bacteria to humans. In another study, **Habeeb and Mahdi** [54] explored the mechanical transmission of bacteria by true fly species and concluded the isolation of 18 different bacterial species; *E. coli*, *Staphylococcus*, *Streptococcus*, and *Enterobacter* from five species of Muscidae, Calliphoridae, and Sarcophagidae.

In diverse locations of Lahore City. According to their findings, acute gastroenteritis seems to be the most significant public health issue. *Musca domestica* serves as a vector host for infectious diseases, they spread helminths, bacteria, protozoa, viruses, and many parasites that are significant to public health [55, 56, 57].

## 5. Conclusion

In conclusion, the present study provides an in-depth analysis of different bacterial species isolated from the outer surface of the flies causing myiasis that were associated with rabbit carcass. A total of 340 adult insects were collected from rabbit carcass during decomposition, most of them are flies. The greatest numbers and highest diversity of flies' species were recorded in fresh and bloated stages of decomposition. Moreover, these flies were highly contaminated with different pathogenic bacteria.

The potential incidence of infective bacteria such as *E. coli* *Klebsiella pneumoniae* ssp. *pneumoniae* *Pseudomonas aeruginosa* and *Proteus mirabilis*, in the flies under study, may be in part responsible for the public health issues that the world currently faces. On the other hand, due to their behavioral traits that assure their contact with human and animal waste, carcasses and food, flies are serving as mechanical vectors in the transfer of the causal agents. Accordingly, the government should take the required efforts to raise awareness among all people about personal hygiene and clean all surfaces subjected to flies. Cleaning and preventing cross-contamination are both essential to avoid bacterial infection.

## Conflict of interest

All authors proclaim that there is no conflict of interest.

## Availability of data

This investigation offers all the data collected or estimated throughout this effort.

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